The Search for Chlorinated Dibenzofurans and Chlorinated Dibenzodioxins in Wildlife Populations Showing Elevated Levels of Embryonic Death

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Introduction

Various kinds of reproductive abnormalities have been documented among wildlife species in North America. All are associated with areas of high agricultural or industrial activity and have been shown, or are assumed to be, pollutant-induced. Selected wildlife populations may therefore serve as the best indicators of the presence in the environment of compounds which have deleterious effects on organisms at very low concentrations, such as several of the chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans.

The best documented of the abnormalities observed in wildlife is the shell thinning of eggs of raptorial and fish-eating species of birds. Contemporary samples show a significant increase in the variance of such parameters as shell weight, shell thickness and an index of shell thickness when compared with samples obtained prior to 1940 (*1*). Most if not all of the increased variance can be explained, however, by a function of the concentration of the DDT compound 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p'*-DDE) (*2*).

Embryonic mortality is reducing the reproductive success of several bird species, including the merlin, *Falco columbarius*, and prairie falcon, *Falco mexicanus*, of western Canada (R. Fyfe, personal communication), the osprey, *Pandion haliaetus*, of the northeastern United States (P. Spitzer, personal communication), the herring gull, *Larus argentatus*, of the Great Lakes region (*3*), and the common terns, *Sterna hirundo*, of Lake Ontario (M. Gilbertson, personal communication). In 1972 virtually no young hatched in the colonies of the latter two species on Lake Ontario (M. Gilbertson, personal communication). The embryotoxicity of commercial PCB has been attributed to chlorinated dibenzofuran contaminants in the preparations (*4–6*). These compounds, associated with or derived from PCB, are therefore a possible cause of the embryonic death observed in wild bird populations. Other

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pollutants which could contribute to the mortality, including the organochlorine insecticides and their derivatives, are invariably present in these samples; some of the mortality could also be an indirect result of the DDE-induced shell thinning.

A low incidence of birth defects has been documented in a colony of common terms in Long Island Sound (*7*). A proportionately larger number were found in 1972 in a common tern colony in Lake Ontario (M. Gilbertson, personal communication). The same kinds of birth defects—primarily those of the beak, eyes and feet—have been produced experimentally by the chlorinated dibenzodioxins (*8*).

The sea lions, *Zalophus californianus,* inhabitating the coastal waters of southern California, have shown an incidence of premature births in recent years which is judged to be substantially higher than the expected normal. A group of females giving birth to premature pups in 1970 had higher levels of both the DDT and PCB compounds than did a group of full term parturient females (*9*). There is no evidence of fetal death in this population; rather, the abnormality consists of a premature onset of partus. It is therefore unlike the fetal mortality and resorption observed among domestic mink, *Mustela vison,* that are fed either coho salmon, *Oncorhynchus kisutch,* obtained from Lake Michigan, or a diet supplemented with the commercial PCB preparation Aroclor 1254 (*10*). The pattern of fetal mortality and resorption observed among the mink is very similar, however, to that found in female rats on a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*11*). PCB levels in the sea lion population of southern California are among the highest found in North American mammals (*9*): they therefore provide favorable material in which to look for the associated chlorinated dibenzofurans.

Chlorinated dibenzodioxins and dibenzofurans were looked for in tissues of marine fish and birds from the Bay of Fundy and were not detected (*12*). The detection limits, however, were substantially above those known to be toxic to either rats (*11*) or chicks (*8*).

In the current study we are examining the hypothesis that several kinds of abnormalities, as yet unexplained, may be caused by chlorinated dioxins or benzofurans that have accumulated in the aquatic food webs of polluted areas; the initial results are reported in the present paper.

Materials and Methods

The initial material selected for examination consisted of a pooled collection of 45 herring gull eggs and pooled samples of sea lion blubber and liver. The herring gull eggs were obtained in the spring of 1972 from Scotch Bonnet island in Lake Ontario. Very few of the eggs laid in this colony have hatched in recent years (M. Gilbertson, personal communication). The sea lion samples had been obtained from nine females that had just given birth to premature pups on San Miguel Island, California, in the spring of 1972, during a study undertaken by the U.S. National Marine Fisheries Service, Seattle, and the Naval Underseas Center, San Diego.

Preparation of Standards

Clophen A-60, a German PCB preparation containing 60% chlorine, was selected as a source of chlorinated dibenzofurans on the basis of the earlier work by Vos et al. (*5*). A subsample of the Clophen A-60 in which the chlorinated dibenzofurans had been detected (Lot No. 912434) was provided to us by J. G. Vos. The first phase of the isolation procedure followed is similar to that outlined by Vos et al. (*5*). A 550-mg portion of the Clophen was dissolved in 400 ml of hexane and added to a Florisil column in 50-ml portions. The Florisil was activated and freed of contaminants by methods previously described (*13*). Each portion was allowed to sink to the surface at an elution rate of 5–10 ml per minute. The Florisil (180 g) was packed in a glass column with a Teflon stopcock and an outside diameter of 44 mm. As the last portion of the PCB solution eluted to the Florisil surface, a 400-

ml portion of hexane was added to the column. The first 50-ml portion was used to rinse the walls and was allowed to elute to the surface as before. The hexane was followed successively by 400-ml portions each of 5% diethyl ether–hexane, 25% diethyl ether–hexane, and acetone. A cleaner preparation of the chlorinated dibenzofurans isolated from Clophen has been found in an acetone fraction after elution with 25% diethyl ether–hexane (J.G. Vos, personal communication).

The diethyl ether and acetone fractions were evaporated just to dryness under a stream of dry nitrogen, and the residue taken up in hexane. This step was repeated twice. Each of the three fractions was then placed on a micro activated alumina column (Fisher alumina, Cat. No. A-540), to further separate the suspected contaminants from PCB (*14*). In order to remove all of the PCB, the volume of the first eluting solvent system, 1% methylene chloride–hexane, was increased to 15 ml, and that of the second, 20% methylene chloride–hexane, to 10 ml. Chlorinated dibenzo-dioxins appear in the latter fraction, and it is probable that the chlorinated dibenzofurans do as well (M.L. Porter and J.A. Burke, personal communication).

This fraction was then evaporated just to dryness, and the residue taken up in hexane. This step was repeated twice. Gas chromatographic analysis and fraction collection for subsequent mass spectrometric identification of components is described below.

Preparation of Environmental Samples

The herring gull eggs were individually weighed, the length and breadth measured for subsequent shell thickness index determination, and the contents pooled and freeze-dried for 5 days. Total wet weight was 3121 g, and total freeze-dried weight 567 g. The dried eggs were ground with sodium sulfate (2:1) in a heavy glass mortar, and an appropriate amount was placed in a glass thimble for extraction in a Soxhlet apparatus (extra-large size, Corning Glass Works No. 415070) fitted to a 2000-ml flask containing 1400 ml hexane–acetone azeotrope. Each portion was extracted for at least 8 hr. This procedure was repeated until the entire sample was extracted. The original solvent was left in the flask and was used for the extraction of all portions. The total extraction period was 119 hr.

The solvent was then concentrated with the use of a rotary evaporator, transferred to a 1000-ml graduated cylinder, and the volume adjusted to 1000 ml with hexane. Lipid determinations were then made. Three 3-ml aliquots were transferred from the cylinder to each of three preweighed aluminum pans (3.0 ml volume in a 5.0-ml disposable pipet), allowed to air-dry, then placed in a 100°C oven for 30 min. After cooling, the dishes containing the lipid were weighed. The total lipid recovered was 250 g.

The clean-up procedure used on the remaining sample extract was adapted from that detailed in Stanley and LeFavoure (*15*). The extract was taken from the graduated-cylinder in four 200-ml portions with a 50-ml volumetric pipet. Each of the four 200-ml portions was passed over 350 g of Celite–sulfuric acid in a Buchner funnel, followed by three 500-ml elutions with hexane. The cleaned extract was concentrated for subsequent fractioning into various chlorinated hydrocarbon components (see below).

The maximum amount of lipid placed on each Davidow column of unit 15 gCelite–9 ml sulfuric acid-9 ml fuming sulfuric acid was kept to 4 g.

Sea lion liver samples from nine females were weighed, pooled, and freeze-dried for 5 days. The total wet weight was 425.1 g, and the total freeze-dried weight was 121.4 g. The dried samples were ground with sodium sulfate (1:8), and a portion placed in a Soxhlet apparatus for extraction. Conditions were similar to those outlined above for herring gull eggs. The total extraction time for all portions was 72 hours. Solvent was concentrated as above, and aliquots taken for lipid determination. The lipid recovery was 16.6 g. The remainder was divided into two portions. Each was passed over 60 g of Celite-sulfuric acid, followed

by three 150 ml elutions with hexane. The cleaned-up extract was concentrated for further fractionation and analysis.

Sea lion blubber samples were from eight of the nine females sampled for liver. The total wet weight was 493 g. Small slices were ground with sodium sulfate (1:6), and the entire mixture was transferred to a Soxhlet apparatus (giant size, Ace Glass, Inc., Vineland, N.J., Cat No. 6810, size H; thimble, Cat. No. 6812, size H). The sample was extracted for 4 days with 6300 ml hexane–acetone azeotrope placed in the 12000-ml flask. The extract was concentrated, aliquots taken for lipid determination, and one quarter of the total was cleaned up and prepared for subsequent fractionation and analysis. The total lipid recovery was 420 g.

Fractionation of Biological Extracts

Each of the three extracts (herring gull eggs, sea lion liver, and sea lion blubber) was divided in two, and each portion was placed on a large Florisil column (180 g) and fractionated by elution with the four solvent systems described for the Clophen, except that the volume of hexane was increased to 800 ml. Subsequent fractionation on the alumina column did not remove enough PCB interference in the 20% methylene chloride eluate. Chlorinated residues from this eluate (dissolved in hexane) were then placed on a smaller activated Florisil column (8 g, 10 mm ID), and eluted with smaller volumes of the same solvent sequence: 150 ml hexane, 200 ml 5% diethyl ether-hexane, 200 ml 25% diethyl ether–hexane, 200 ml acetone. These fractions, evaporated to dryness and taken up in hexane, were then placed on alumina columns and chlorinated hydrocarbon components separated as before.

Gas Chromatography

Extracts were analyzed with the use of an electron-capture (EC) ^{63}Ni Tracor MT-220 gas chromatograph. The stationary phase was 3% OV-1 on 100-120 mesh Supelcoport, placed in a 6 ft × 4 mm. I D Pyrex column. Other operating parameters included: oven temperature, 200° C; inlet temperature, 225° C; detector temperature, 285° C; column flow rate 60 cc/min; purge flow rate, 50 cc/min; carrier and purge gas, nitrogen.

Several of the extracts were further fractionated by collection of individual peak components with the use of a 10:1 splitter (Western Scientific, Danville, California). Glass capillary tubes, bent to a V-shape, were immersed in a beaker of liquid nitrogen during collection of the peak components through a septum.

Mass Spectrometry

The samples were dissolved in 5 μl of methylene chloride and transferred onto the direct inlet probe of the mass spectrometer, where the solvent was allowed to evaporate. The probe was rapidly introduced into the ion source and multiple scans were recorded. Both low and high resolution mass spectrometric analyses were carried out on a GEC-AEI MS-902 mass spectrometer. For high resolution the instrument was used on line to an XDS Sigma 7 computer as described by Burlingame (*16, 17*) and Burlingame et al. (*18*). The ion source operating conditions were: resolution, 10,000; ionizing current, 500 μA; ionizing voltage, 50 eV; temperature, 200–220°C. The scan rate was 16 sec per decade with a clock rate of 24 kHz. Multiple scans were taken during each analysis and then sum-averaged together during data reduction. For low resolution the instrument was scanned either on line to an XDS Sigma 2 computer (*19*) or manually with an ultraviolet-sensitive strip-chart recorder.

Results and Discussion

The pooled sample of herring gull eggs was found by GC-electron capture analysis to contain 35 ppm DDE and 300 ppm PCB on a wet weight basis or 440 ppm and 3700 ppm, respectively, on a lipid basis. The PCB content was therefore approximately 0.9 g. The sea lion liver sample contained 12 ppm DDE and 3 ppm PCB on a wet weight basis or 300 ppm and 80 ppm, respectively, on a lipid basis. The PCB content of the sample was therefore approximately 1 mg. DDE

and PCB concentrations in the blubber were 512 and 62 ppm on a wet weight basis, 630 and 76 ppm on a lipid basis. The PCB content of the entire sample was therefore 30 mg, of which 7.5 mg was used for the extraction process.

The electron capture chromatogram of the 20% methylene chloride–hexane extract of Clophen A-60 is shown in Figure 1. The components of peaks A–G were collected for individual analysis by low resolution mass spectrometry. Retention times of the peaks relative to dieldrin, and the identity of the compounds identified are listed in Table 1. All of the peaks were identified as chlorinated dibenzofurans; no other significant components were detected. Compounds having the same retention time relative to dieldrin as peaks A and C have previously been identified by Vos et al. (*5*) as tetrachloro- and pentachlorodibenzofuran, respectively. In the present study, peak B, consisting of two components on the EC gas chromatogram, was identified as tetrachlorodibenzofuran,

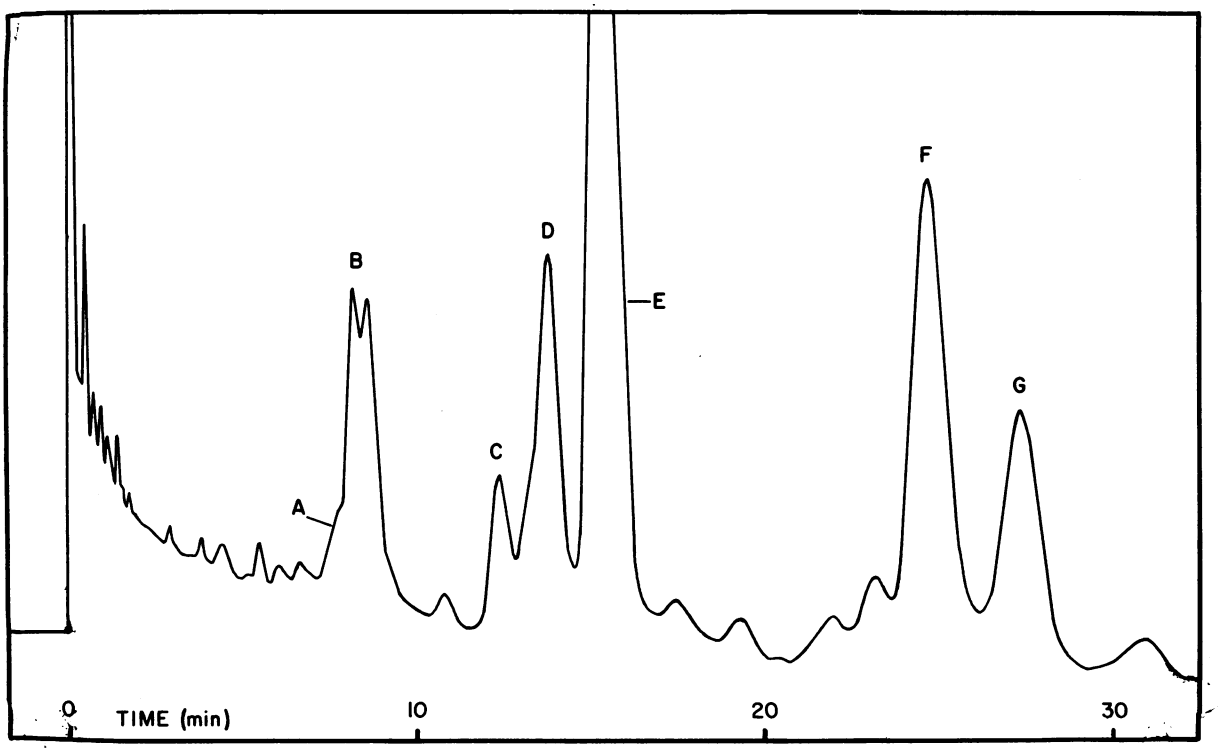

FIGURE 1. Gas chromatogram 3% OV–1 of Clophen A–60, 20% methylene chloride-hexane fraction.

Table 1. Relative retention times and identification of chromatograph peaks shown in Figure 1.

Peak	Retention time, relative to dieldrin	Composition	Mass	
A	1.49	$C_{12}H_4O^{35}Cl_4$	304	Tetrachlorodibenzofuran
B	1.60	"	"	" "
C	2.38	$C_{12}H_3O^{35}Cl_5$	338	Pentachlorodibenzofuran
D	2.64	"	"	" "
E	2.95	"	"	" "
F	4.73	$C_{12}H_2O^{35}Cl_6$	372	Hexachlorodibenzofuran
G	5.24	"	"	" "

probably isomers of peak A. In addition, peaks D and E were identified as pentachlorodibenzofurans and F and G as hexachlorodibenzofurans. Representative mass spectra of a tetra- (B), penta- (E), and hexachlorodibenzofuran (F) are shown in Figure 2. Tetrachlorodibenzofuran fragments were as follows (cf. Fig. 2*a*): the molecular ion M^+

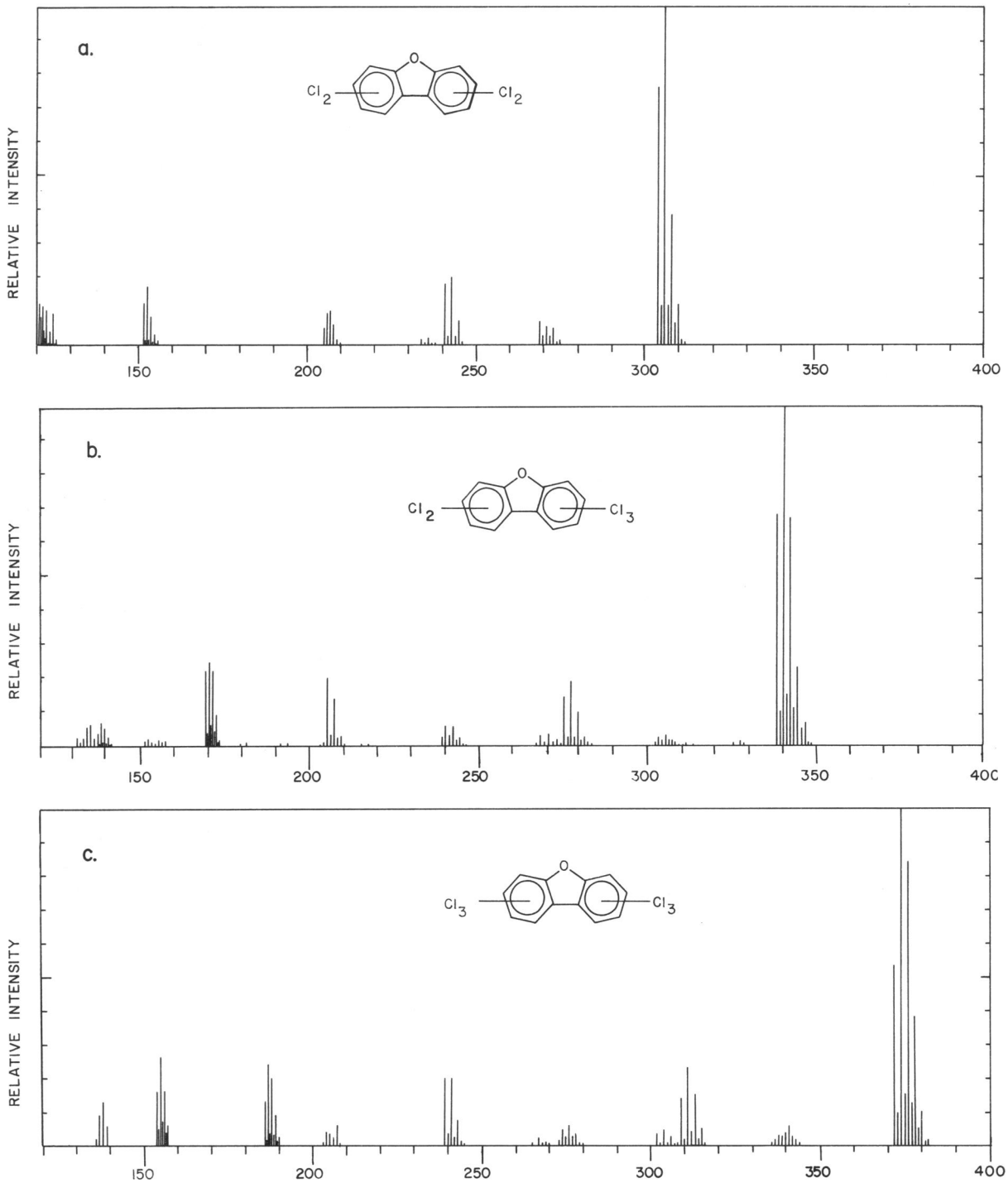

FIGURE 2. Low-resolution mass spectra of compounds isolated from Clophen A–60: (*a*) tetrachlorodibenzofuran; (*b*) pentachlorodibenzofuran; (*c*) hexachlorodibenzofuran.

is found at *m/e* 304–312, loss of Cl yields the peaks at *m/e* 269–275, subsequent elimination of CO results in *m/e* 241–245 and double Cl loss yields the minor peaks at *m/e* 234–238, which on loss of CO and CHO yield the group at *m/e* 205–210. The doubly charged species of the molecular ion are found at *m/e* 152–156, and the strong loss of Cl followed by CO is confirmed by the doubly charged peaks at *m/e* 120–122. The pentachlorodibenzofuran and the hexachlorodibenzofuran fragment were identified in an analogous fashion as described above (cf. Figs. 2*b* and *c*, respectively).

The elemental composition of these compounds was confirmed by high-resolution mass spectrometric analysis (*20, 21*) of the total mixture. Peaks of the homologous series $C_{12}H_{8-n}OCl_n$ were only detected for $n=4$–6, and other halogenated compounds (e.g., chloronaphthalenes) were present as trace constituents.

Sixteen major peaks were present on the EC chromatogram of the second 5% diethyl ether–hexane fraction of the gull eggs, derived from the first 5% diethyl ether–hexane fraction eluted from the large Florisil column. Heights of these peaks were comparable to those of the Clophen A-60 peaks shown in Figure 1. Extract volumes and volumes injected into the gas chromatograph were also comparable. This extract was therefore chosen for analysis by low resolution mass spectrometry. The components of all 16 peaks were isolated. Retention times relative to dieldrin on the OV-1 column were: A, 1.38; B, 1.56 and 1.73; C, 2.14; D, 2.32; E, 2.52; F, 2.72; G, 3.14; H, 3.66; I, 4.21; J, 4.65; K, 4.98; L, 5.20; M, 5.95; N, 6.75; and O, 8.20. No chlorinated dibenzofurans were detected. The majority of the peaks represented chlorine-containing compounds with mass numbers from 362 to 508 that have not yet been identified.

Peak G was subjected to high-resolution mass spectrometric analysis (HRMS) and was found to be mainly a hexachloronaphthalene ($C_{10}H_2{}^{35}Cl_6$, *m/e* 331.8252; $C_{10}H_2{}^{35}Cl_5{}^{37}Cl$, *m/e* 333.8275; $C_{10}H_2{}^{35}Cl_4{}^{37}Cl_2$, *m/e* 335.8202) Fragment ions due to loss of two Cl where also present in these data.

Peak N was also subjected to HRMS analysis. A group of intense peaks was observed at *m/e* 436.8744, 438.8743, 440.8758, 442.8733, and 444.8622. The compound structure has not yet been identified.

The entire 5% diethyl ether–hexane fraction of the gull eggs that had been derived from the initial acetone fraction was examined by HRMS. No chlorinated dibenzofuran or dibenzodioxin compounds were detected.

None of the appropriate sea lion liver extracts produced EC peaks of sufficient height to merit analysis by mass spectrometry. Components of individual peaks of the 5% diethyl ether–hexane fraction of the initial acetone fraction, and of the 25% diethyl ether–hexane fraction derived from the initial 25% diethyl ether–hexane fraction of the sea lion blubber, were isolated for low resolution mass spectrometry; a portion of the latter extract was also analyzed by HRMS. No chlorinated compounds of interest were detected, in part because of the high hydrocarbon content of the samples.

A more exhaustive examination of these and other wildlife samples is evidently required to permit a conclusion about the presence of either chlorinated dibenzodioxins or chlorinated dibenzofurans in the North American environment.

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REFERENCES

1. Anderson, D. W., and Hickey, J. J. Eggshell changes in certain North American birds. Proc. XV Int. Ornith. Cong.: 433 (1972).
2. Risebrough, R. W. Effects of environmental pollutants upon animals other than man. In Proceedings Sixth Berkeley Symposium Mathematical Statistics and Probability, L. M. Lecam, J. Neyman, and E. L. Scott, Eds., University of California Press, Berkeley–and Los Angeles, 1972, p. 443.
3. Keith, J. A. Reproduction in a population of Herring Gulls (*Larus argentatus*) contaminated by DDT. J. Appl. Ecol. (Suppl.) 3: 57 (1966).
4. Vos, J. G., and Koeman, J. H. Comparative toxicologic study with polychlorinated biphenyls in chickens with special reference to porphyria, edema formation, liver necrosis and tissue residues. Toxicol. Appl. Pharmacol. 17: 656 (1970).
5. Vos, J. G., et al. Identification and toxicological evaluation of chlorinated dibenzofuran and chlorinated naphthalene in two commercial polychlorinated biphenyls. Food Cosmet. Toxicol. 8: 625 (1970).
6. Vos, J. G. Toxicology of PCBs for mammals and for birds. Environmental Health Perspect. No. 1: 105 (1972).
7. Hays, H., and Risebrough, R. W. Pollutant concentrations in abnormal young terns from Long Island Sound. Auk 89: 19 (1972).
8. Verrett, J. Statement before the Subcommittee on Energy, Natural Resources, and the Environment of the Committee on Commerce, United States Senate, Ninety-first Congress. Second Session on Effects of 2,4,5-T on Man and the Environment. Serial 91–60, U.S. Government Printing Office, 1970, p. 190.
9. DeLong, R. L., Gilmartin, W. G., and Simpson, J. G. Premature births in California sea lions associated with high organochlorine pollutant residue levels. Science, in press.
10. Ringer, R. K., Aulerich, R. J., and Zabik, M. Effect of dietary polychlorinated biphenyls on growth and reproduction of mink. Paper presented at 164th National Meeting, American Chemical Society, Division of Water, Air and Waste Chemistry, New York City, August 28–September 1, 1972.
11. Sparschu, G. L., Dunn, F. L., and Rowe, V. K. Study of the teratogenicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in the rat. Food Cosmet. Toxicol. 9: 405 (1971).
12. Zitko, V., Hutzinger, O., and Choi, P. M. K. Contamination of the Bay of Fundy-Gulf of Maine area with polychlorinated biphenyls, polychlorinated terphenyls, chlorinated dibenzodioxins, and dibenzofurans. Environ. Health Perspect. 1: 47 (1972).
13. Bowes, G. W., and Lewis, J. A. Extraction of polychlorinated biphenyls (PCB): evaluation or a column technique applied to polar bear and seal tissue. J. Assoc. Offic. Anal. Chem., in press.
14. Porter, M. L., and Burke, J. A. Industrial chemicals: separation of three chlorodibenzo-*p*-dioxins from some polychlorinated biphenyls by chromatography on an aluminum oxide column. 54: 1426 (1971).
15. Stanley, R. L., and LeFavoure, H. T. Rapid digestion and cleanup of animal tissues for pesticide analysis. J. Assoc. Offic. Anal. Chem. 48: 666 (1965).
16. Burlingame, A. L. Data acquisition, processing and interpretation via coupled high-speed real-time digital computer and high resolution mass spectrometer systems. In: Advances in Mass Spectrometry, Vol. 4, E. Kendrick, Ed., The Institute of Petroleum, London, 1968, p. 15.
17. Burlingame, A. L. Developments and applications of real-time high resolution mass spectrometry. In: Recent Developments in Mass Spectroscopy, K. Ogata and T. Hayakaw, Eds., University of Tokyo Press, Tokyo, 1970, p. 104.
18. Burlingame, A. L., et al. Real-time high resolution mass spectrometry. In: Computers in Analytical Chemistry: Progress in Analytical Chemistry, Vol. 4, C. H. Orr and J. A. Norris, Eds., Plenum Press, New York, 1970, p. 17.
19. Smith, D. H., et al. Real-time organic mass spectrometry: LOGOS—a general laboratory system for high and low resolution GC–MS and closed-loop applications. Anal. Chem. 43: 1796 (1971).
20. Simoneit, B. R., et al. Application of real-time mass spectrometric techniques to environmental organic geochemistry. I: General considerations. Arch. Environ. Contamin. Toxicol. in press.
21. Simoneit, B. R., et al. Application of real-time mass spectrometric techniques to environmental organic geochemistry. II: San Francisco Bay area water. Arch. Environ. Contam. Toxicol. in press.